Recombinant *Bacillus thuringiensis* Crystal Proteins with New Properties: Possibilities for Resistance Management

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To obtain *Bacillus thuringiensis* crystal proteins with new properties and to identify the regions involved in insecticidal activity, we generated hybrid genes composed of *cryIC* and *cryIE* by *in vivo* recombination. Analysis of the hybrid proteins showed that domain III of CryIC is involved in the toxicity towards *Spodoptera exigua* and *Mamestra brassicae*. Transfer of this domain to CryIE, which is not active against these insects, resulted in a new protein with a broader activity. This hybrid protein binds to different receptors than CryIC, suggesting its use as an alternative for CryIC in resistance management programs.

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Bacillus thuringiensis produces a number of proteins that accumulate as crystals, and are toxic to a variety of insect larvae. Crystal proteins (Cry) can be grouped into different classes based on sequence homology and insecticidal specificity[1], and, as deduced from the 3-D structure of CryIIIA, their toxic moiety is composed of three distinct structural domains. Domain I, the most N-terminal, consists of 7 α-helices. Domain II comprises of 3 β-sheets and domain III folds into a β-sandwich[2].

The mode of action of crystal proteins has been partially elucidated. After oral uptake, the crystals dissolve in the alkaline environment of the larval midgut and the protoxins are processed by midgut proteases to a 65 kD protease-resistant toxic fragment[3]. The toxic fragment subsequently binds to receptors on epithelial cells of the midgut[4,5] and penetrates the cell membrane. This eventually leads to lysis of these cells and death of the larvae. Binding studies with purified toxins and vesicles prepared from midguts have demonstrated that the presence of receptors for a specific crystal protein is essential for toxicity and that in one insect, different receptors for different crystal proteins can be present[4-6].

The importance of binding to midgut epithelial receptors is clear from cases in which insects that developed resistance to one of the crystal proteins showed significantly reduced binding of this protein to midgut epithelial cells while the binding and toxicity of other crystal proteins remained unaffected[6,7]. To anticipate the development of resistant insects, we have generated proteins with new properties by combining the insecticidal proteins CryIC and CryIE. Both are toxic to lepidopterans but have different specificities. CryIC is particularly active against *S. exigua*[8,9] and *M. brassicae*[10] whereas both proteins are active against *M. sexta*[3].

Results

Construction of hybrid crystal proteins. Hybrid *cryI* genes were constructed by *in vivo* intramolecular recombination utilizing two tandem plasmids. These plasmids each carry two truncated crystal protein genes that overlap only in domains II and III, thus limiting homologous recombination to these regions. In-frame recombinations, which can be selected by restriction enzyme digestion prior to transformation, generate plasmids that express full size 140 kD hybrid protoxins (Fig. 1).

Tandem plasmids pBD560 and pBD650 generate cryIC-cryIE and the reciprocal cryIE-cryIC hybrids respectively. The plasmids were allowed to recombine in an *E.coli recA*+ back-

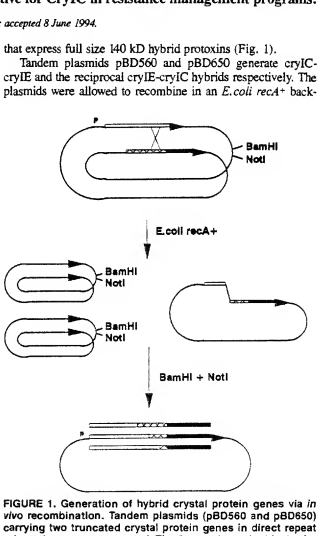

FIGURE 1. Generation of hybrid crystal protein genes via *in vivo* recombination. Tandem plasmids (pBD560 and pBD650) carrying two truncated crystal protein genes in direct repeat orientation were constructed. The 5' gene (open bar) lacks the protoxin encoding region (solid bar) and in the 3' gene (dashed bar) part of the domain I encoding region is deleted. *In vivo* recombination between homologous regions (domain II and III) occurs in a *recA*- background. Selection against non-recombinants by digestion with NotI and BamHI and subsequent transformation resulted in a set of plasmids encoding hybrid crystal proteins.

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                                                     Domain              G27                 H8                       H17
                                                    II←  →III             ↓                   ↓                        ↓
CryIE  (420) vgtshrlshvtltrslyntnltslptfvwth  HSATNTNTINPDIITQIPLVKGFRLGGGTSVIKGPGFTGGDILRRNTIGEFVSLQVNINSPITQRYRLRFRYAS
CryIC  (423) s......c.a.fvqrsgtpfl.tgvv.s...  R...L....D.ER.N.........VW......T..............F.D.......................
                ↑    ↑                                 ↑
               F59  F71                               F26

                                                 H13          H7                                   H21
                                                  ↓            ↓                                    ↓
CryIE  (525) SRDARITVAIG-------GQIRVDMTLEKTMEIGESLTSRTFSYTNFSNPFSFRANPDIIRIAEELP-----IRGGELYIDKIELILADATFEEEYDLERAQK  (615)
CryIC  (528) .....VI.LT.AASTGVG..VS.N.P.Q.......N......R..D..............G.S.QPLFGAGS.SS.........I........A.S.......  (630)
                                                                                        ↑
                                                                                        E7
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FIGURE 2. Alignment of amino acid residues 420 to 630 of CryIE and CryIC. The border between domain II and III is indicated. Only amino acids of CryIC which differ from CryIE are given, identical residues are indicated by a dot. The positions of cross-over in CryIE-CryIC hybrids are given above of the alignment and the cross-over of CryIC-CryIE hybrids underneath the alignment.

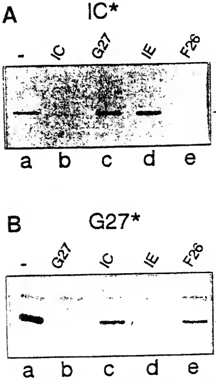

FIGURE 3. Heterologous competition experiments. Biotinylated CryIC (panel A) and G27 (panel B) were incubated with *S. exigua* BBMV vesicles in the absence (lanes a) or presence of an excess of unlabeled protein as indicated. After the incubation, the vesicles were washed, loaded on a SDS-polyacrylamide gel and blotted to a nitrocellulose membrane. Biotinylated crystal proteins, re-isolated with the vesicles, were visualized using streptavidin-peroxidase conjugate and are indicated with an arrow head.

ground, after which plasmid DNA was isolated, digested and transferred to a *recA*⁻ strain. For each tandem plasmid, 100 colonies from 20 independent experiments were analyzed by SDS-PAGE. Approximately 90% of these clones produced a 140 kD protein indicating in-frame recombination between the truncated *cryI* genes.

When produced in *E.coli*, CryI proteins are isolated as crystals that can be solubilized at high pH *in vitro*. However, although all hybrids could be isolated as inclusion bodies, the majority of these could not be solubilized at high pH (data not shown). The observation that these insoluble hybrids were not toxic towards larvae of *M. sexta* (data not shown), an insect susceptible to both parent proteins, suggests that these were aberrant folding products that precipitated in *E. coli* as biologically inactive aggregates.

Approximately 15% of the CryIC/CryIE hybrid proteins could be solubilized at high pH. The latter were first classified by their point of cross-over using restriction enzymes. Subsequently the precise cross-over point of selected hybrids was determined by sequence analysis. All cross-overs which resulted in soluble hybrid proteins occurred in, or very close to, domain III (Fig.2).

Isolation and biochemical analysis of 65 kD toxic fragments. CryIC, CryIE and the hybrid proteins were purified from *E.coli* as described previously[12,13]. Isolated crystals were solubilized at high pH and incubated with trypsin. Like CryIC and CryIE, all soluble hybrids with cross-overs in domain III were converted to stable 65 kD fragments that could be purified using anion exchange chromatography under similar conditions as the parent proteins (data not shown). These results suggest that these hybrids fold into a conformation comparable to the parental proteins. Hybrids F59 and F71, both with cross-overs in domain II, were completely degraded by trypsin. Apparently, although these hybrids do not precipitate as insoluble aggregates, trypsin cleavage sites buried in the parent proteins become exposed.

Insecticidal activity of *cryIC/cryIE* hybrid gene products. *M. sexta* is susceptible to both CryIC and CryIE. To select functional recombinants, *E. coli* expressing the various hybrids were tested for toxicity against this insect. As expected from their trypsin sensitivity, cells expressing the domain II hybrids, F59 and F71, were not active. Although domain III hybrids H13 and H7 could be converted to stable 65 kD proteins by trypsin, cells expressing these proteins were also not toxic to *M. sexta*. All remaining hybrids produced a stable 65 kD fragment toxic to *M. sexta*, and only these were further analyzed.

The 65 kD fragment of CryIC is highly toxic towards *S. exigua* and *M. brassicae* whereas that of CryIE is not. The relative activity of selected hybrid toxic fragments towards *S. exigua* and *M. brassicae* larvae was determined (Table 1). The 65 kD fragment of G27, a CryIE-CryIC hybrid with a cross-over at the junction of domain II and III, is highly active against

both insects. Thus domain III of CryIC is sufficient to confer full activity against *S. exigua* and *M. brassicae* to CryIE. Hybrid H8, which differs by only three amino acids from G27, is significantly less active against *S. exigua*.

F26, the reciprocal hybrid of G27, in which domain III of CryIC has been exchanged with domain III of CryIE, although toxic to *M. sexta*, has no activity against *S. exigua* and *M. brassicae*. Only when CryIC sequences to amino acid residue 602 are present in the hybrid (E7), is activity towards these insects restored.

Receptor binding characteristics of selected hybrids. Since binding to epithelial gut cells is a key step in the mode of action of crystal proteins, the binding of biotinylated crystal proteins to *S. exigua* brush border membrane vesicles was investigated. Binding of labeled CryIC (Fig. 3A, lane a) and labeled F26 (not shown) can be eliminated by an excess of unlabeled CryIC (lane b) or F26 (lane e), but not with an excess of G27 (lane c) or CryIE (lane d). Furthermore, binding of labeled G27 (Fig. 3B, lane a) and labeled CryIE (not shown) can be eliminated by an excess of G27 (lane b) or CryIE (lane d), but not with an excess of CryIC (lane c) or F26 (lane e). Thus G27 and CryIE recogize the same binding sites on *S. exigua* midgut membranes and these sites differ from those recognized by CryIC and F26.

Discussion

The availability of multiple crystal proteins active against the same insect species, but with different receptor binding characteristics, may be important in the longterm application of crystal proteins in pest control.

Although CryIE was previously reported to be active against *S. exigua*[9], the toxicity of CryIC against *S. exigua*[5] (this study) and *M. brassicae*[10] is much higher and more consistent (see below). Amino acid sequence comparison of CryIC and CryIE reveals a limited homology in domain II and a much higher homology in domain III[10]. It is tempting to correlate regions with low sequence homology to differences in insecticidal specificity, and those with high sequence homology to overlapping insecticidal activity. This is in agreement with domain II being directly involved in receptor binding, as suggested by the 3-D structure of CryIIIA[2], and with receptor binding playing a crucial role in the activity spectrum of crystal proteins. Indeed, insect specificity and receptor binding has been repeatedly shown to reside in domain II[4–8].

Surprisingly, in this study we found that, although receptor binding follows domain II, it is domain III which is involved in the specificity of CryIC towards *S. exigua* and *M. brassicae*. The toxic fragment of G27, a hybrid composed of domains I and II of CryIE and domain III of CryIC is, in contrast to CryIE, highly active against these insects. Receptor binding experiments showed that CryIC and CryIE bind to different sites on midgut membranes of *S. exigua*. The G27 hybrid binds to the same sites as CryIE, and the F26 hybrid to those recognized by CryIC. Since G27 and CryIC are equally effective against *S. exigua* but bind to different receptors, G27 might be used in resistance management programs as an alternative to CryIC, one of the few known crystal proteins active against this insect. The precise reasons for the unique properties of G27 are not clear. One explanation might be that although domain II of CryIE can bind to *S. exigua* and *M. brassicae* midgut receptors, domain III of CryIC is needed to stabilize the toxic fragment in the gut of these insects. This might explain the inconsistency reported for CryIE activity against *S. exigua*[9,10] (this study) since different purification and/or application methods could affect its (proteolytic) inactivation. In G27 such sites would be replaced by the corresponding more stable regions of CryIC. This predicts that other crystal proteins can be made active against formerly insensitive insects by providing them with new domain III sequences.

TABLE 1. Relative toxicity of CryIC, CryIE and hybrid toxic fragments.

Toxic fragment	Activity (EC_{50} in ng/cm²)[a] against *S. exigua*		*M. brassicae*	
CryIC	+ +	(26 ± 9)	+ +	(8 ± 4)
CryIE	-	(> 1000)	-	(> 1000)
G27	+ +	(20 ± 7)	+ +	(12 ± 6)
H8	+	(80 ± 15)	nd	
H21	-		-	
F26	-	(> 1000)	-	(> 1000)
E7	+ +		+ +	

[a]When three or more independent experiments were performed, the EC_{50} (concentration giving 50% growth reduction) is given as the mean of these experiments between brackets. nd: not determined.

Experimental Protocol

Bacterial strains and plasmids. *Escherichia coli* XLI-blue (Stratagene Inc.) was used as plasmid host except in cases were JM101 was used to provide a recombination proficient background. A vector for the expression of crystal proteins in *E. coli* was derived from pKK233-2 (Pharmacia LKB Biotechnology). The size of pKK233-2 was reduced by deleting an EcoRI-PvuII fragment carrying the gene encoding tetracycline resistance. Subsequently a 6 bp XhoI linker was ligated into the HindIII site resulting in pBD10. Plasmid BK + was created by insertion of a BglII linker in the SacI site of Bluescript SK + (Stratagene Inc.). Subsequently, the polylinker of BK + from BglII to XhoI was introduced in pBD10. The resulting expression vector, designated pBD11, contains the highly expressed *trc* promoter, the *lacZ* ribosome binding site and ATG initiation codon. The initiation codon overlaps with a NcoI site and is followed by the polylinker to facilitate insertions into the vector. Transcription is terminated by the *rrnB* transcription terminator. The cloning of *cryIC* and *cryIE* from *B. thuringiensis* subsp. *entomocidus* 60.5 and *kenya* 4F1 respectively, has been described previously[4,10]. A DraI fragment from pEM4 (ref. 10) containing the complete *cryIE* gene was cloned in the EcoRV site of SK +, resulting in plasmid pEM15. By *in vitro* mutagenesis, NcoI sites were created overlapping with the start codons of *cryIC* and *cryIE* and a BglII site was created directly downstream the translation termination codon of *cryIC*, resulting in the sequence ATAAGATCTGTT (stopcodon underlined). The NcoI-BglII and NcoI-XhoI fragments containing the *cryIC* and *cryIE* coding regions respectively, were ligated into pBD11, resulting in CryIC and CryIE expression plasmids pBD150 and pBD160 respectively. From pBD150 a derivative, pBD155, was constructed in which the polylinker sequences 3' of *cryIC* have been deleted. Convenient plasmids carrying only toxic fragment encoding regions of the *cryI* genes were constructed. BglII linkers were ligated into the XmnI site present at bp 1835 of *cryIC* and into the HgiAI site at position 1839 of *cryIE* respectively. Subsequently, NcoI-BglII fragments containing the *cryIC* (1835 bp) and *cryIE* (1839 bp) toxic fragment encoding regions were ligated into pBD11 resulting in pBD151 and pBD161 respectively. The tandem plasmid used for the generation of *cryIC-cryIE* hybrid genes were constructed as follows. BamHI linkers were ligated to pBD151 digested with HpaI. This DNA was incubated with BamHI and XhoI and the truncated *cryIE* gene running from bp 704 was ligated 3' to the CryIC toxic fragment encoding sequence in pBD151 resulting in pBD560. To construct a tandem plasmid for the generation of *cryIE-cryIC* hybrids, pBD155 was digested with NsiI and XhoI. The fragment carrying the truncated *cryIC* gene running from bp 266 was ligated 3' to the CryIE toxic fragment encoding sequence in pBD161, resulting in plasmid pBD650. Due to polylinker sequences, unique NotI and BamHI restriction enzyme sites are present between the two truncated *cryI* genes present on the tandem plasmids pBD560 and pBD650.

DNA manipulations. All recombinant DNA techniques were as described by Sambrook et al.[11]. DNA sequencing was performed by the dideoxyribophosphate method with fluorescent dyes attached to the dideoxynucleotides. Analysis was automated by using an Applied Biosystems 370A nucleotide sequence analyzer. To generate *in vivo* recombinants, a tandem plasmid (either pBD560 or pBD650) was transferred to JM101. Per experiment, DNA from a single colony was isolated, and 5 µg was digested with NotI and BamHI, cutting between the two truncated *cryI* genes to select against non-recombinants, and the DNA was transformed to *E. coli* XL1-blue. Per independent transformation, 5 single colonies were grown and protein patterns and plasmid content were analyzed.

Protein manipulation and analysis. Crystal proteins were isolated solubilized at pH 10 and activated with trypsin essentially as described[12,13]. The 65 kD toxic fragments were subsequently purified on a Mono Q 5/5 column connected to a fast-protein liquid chromatography (FPLC) system (Pharmacia LKB Biotechnology). Biotinylation of crystal proteins was performed using biotin-N-hydroxysuccinimide ester essentially as

described by the manufacturer (Amersham). One mg of crystal protein was incubated with 40 µl biotinylation reagent in 50 mM NaHCO3, 150 mM NaCl pH8.6 for one hour at 20°C. The solution was loaded on a Sephadex 25 column equilibrated with the same buffer containing 0.1 % BSA to remove unbound biotin. Samples of the fractions were spotted on a nitrocellulose membrane and fractions containing biotinylated crystal proteins were visualized using streptavidine-peroxidase conjugate and pooled. Proteins were separated by 7.5 % SDS-PAGE.

Insect toxicity assays. Bacterial cultures were concentrated to OD_{660} 6.0 and 100 µl was spotted on 2 cm^2 of artificial diet in a 24-well tissue culture plate. Alternatively, diluted samples of purified toxic fragments were applied to the diet. Second instar larvae of either *S. exigua*, *M. brassicae* or *M. sexta* were fed on these diets (16 per sample dilution) for 5 days, after which the larval weight was scored. The relative growth (EC_{50}, the concentration giving 50 % growth reduction) was determined by calculating the ratio between the mean weight of larvae grown on diet supplemented with toxin and the mean weight of control larvae grown on a diet without toxin. Each experiment was performed at least in duplicate (see Table 1). *M. sexta* egg layers were supplied by Carolina Biological Supply Company (North Carolina, US).

Binding assays. Brush border membrane vesicles (BBMV) were isolated as described by Wolfersberger et al[14], except that the vesicles were washed one additional time with isolation buffer with 0.1 % Tween-20. Binding of biotinylated crystal proteins to brush border membrane vesicles was performed in 100 µl PBS, 0.1 % BSA, 0.1 % Tween-20 pH7.6. Vesicles (20 µg vesicle protein) were incubated with 10 ng biotinylated crystal proteins in the presence (or absence) of 1000 fold excess of unlabeled crystal proteins for 1 hour at 20°C. Subsequently, the vesicles were reisolated by centrifugation for 10 minutes at 14000 g in an eppendorf centrifuge, washed twice with binding buffer, resuspended in sample buffer, denatured by heating and loaded on 7.5 % SDS-polyacrylamide gels. After electrophoresis, proteins were blotted to nitrocellulose membranes and biotinylated crystal proteins, which were re-isolated with the vesicles were visualized by incubation with streptavidin-peroxidase conjugate and luminol (ECL, Amersham).

Acknowledgments

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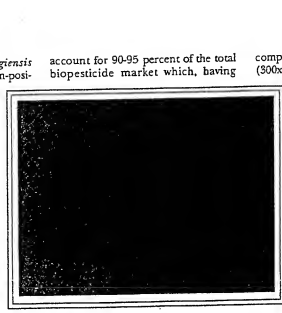
BY JERALD S. FEITELSON, JEWEL PAYNE, & LEO KIM

Bacillus thuringiensis: Insects and Beyond

Bacillus thuringiensis (*B.t.*) is a Gram-positive, spore-forming bacterium characterized by parasporal crystalline protein inclusions. These often appear microscopically as distinctively shaped crystals (see photomicrograph). The proteins are highly toxic to pests and specific in their activity. Over the past 30 years, commercial use of *B.t.* pesticides has been largely restricted to a narrow range of Lepidopteran (caterpillar) pests. In recent years, however, investigators have discovered *B.t.* pesticides with specificities for a much broader range of pests. The toxin genes have been isolated and sequenced, and recombinant DNA-based *B.t.* products produced and approved. Many of the newly discovered strains have activities that would extend the use of *B.t.* beyond traditional agricultural markets.

Consequently, the producers of *B.t.* pesticides and the owners of patent rights to the genes anticipate rapid expansion in their markets. *B.t.* products account for 90-95 percent of the total biopesticide market which, having



grown from $24M in 1980 to $107M in 1989, is forecast to expand at an annual rate of 11 percent to reach $300M by 1999[1]. The availability of a large number of diverse *B.t.* toxins may also enable farmers to adopt product-use strategies that minimize the risk that *B.t.*-resistant pests will arise.

B.t. BASICS

Commercial *B.t.*-based bioinsecticides, used worldwide for controlling the larval forms of economically important pests, are usually formulations of the spores and crystalline inclusions that are released upon lysis of *B.t.* during its stationary phase of growth. The products are applied at 10-50 g acre^{-1} or about 10^{20} molecules acre^{-1}. The molecular potency of *B.t.* toxins is high compared to that of other pesticides (300x higher than synthetic pyrethroids, for instance, which are applied at about 3×10^{22} molecules acre^{-1} or 80,000 times higher than organophosphates, which are applied at about 8×10^{24} molecules acre^{-1}).

Much is known about the mechanisms of *B.t.* toxin activity and specificity[2]. The crystalline inclusions initially dissolve in the insect midgut, releasing one or more proteins of 27-140 kDa. Most crystal proteins are protoxins that must be activated by proteolytic cleavage into toxic polypeptides. The activated toxins bind to the cell membrane lining the gut, generating pores that disturb osmotic balance and lead to cellular swelling and lysis. Intoxicated insect larvae quickly stop feeding and eventually die. There is increasing biochemical evidence for the presence of specific high-affinity binding sites on the midgut epithelium that may differ between susceptible and resistant insects[3].

There are two major stimuli for extending the use of *B.t.* pesticides. First, there is an increasing demand for the biological control of pests and, second, traditional *B.t.*-based products are relatively unstable in the environment. In responding to these stimuli, various companies (Table 1) are expressing *B.t.* genes in a number of different systems. Ecogen Corporation (Langhorne, PA) has used conjugal mating to transfer the large mobilizable plasmids on which

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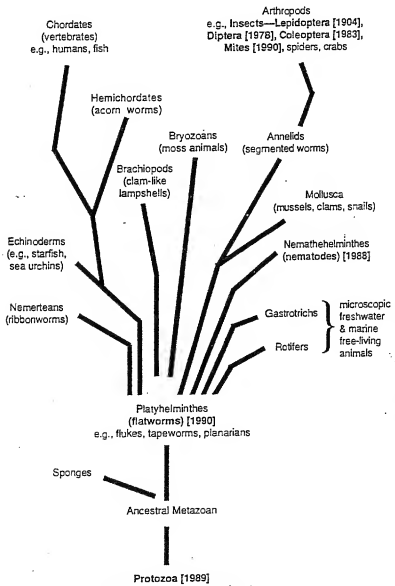
the toxin genes reside to other *B.t.* strains in order to broaden their host range. Crop Genetics International (Hanover, MD), on the other hand, has introduced the toxin gene into an endophytic bacterium. This colonizes the xylem of plants and provides a type of systemic immunity against susceptible insect pests (InCide). But the largest number of small firms and large agrichemical companies have transferred and expressed *B.t.* toxin genes in the tissues of agronomically important plants as part of more general germplasm improvement programs (see the sidebar "Plant or Bacterial *B.t.* ?").

At this point, however, the only approved recombinant-DNA *B.t.* products are Mycogen Corporation's (San Diego, CA) MVP and M-Trak, which were approved for marketing in the U.S. by the Environmental Protection Agency in July, 1991. In these products, the *B.t.* toxin has been, in effect, encapsulated within stabilized *Pseudomonas fluorescens* (*P.f.*) cells[4]. Toxin genes from *B.t.*, introduced into *P.f.* and expressed at high levels, formed inclusion bodies similar to those in *B.t.*. However, unlike *B.t.*, the *P.f.* cells neither lyse nor sporulate during the stationary growth phase. A chemical fixative added to the final fermentation broth rapidly kills and stabilizes the cells by strengthening the cell wall and inactivating proteolytic enzymes. The encapsulated toxin remains active. Field trials indicate that products made through this encapsulation process (CellCap) persist longer than conventional *B.t.* products[5]. Since the organisms are dead and cannot spread from the site of application, the products also create fewer environmental concerns.

Figure 1. The evolutionary spectrum of *B.t.* toxins. The pest targets against which B.t. strains are known to be active are shown in color. How much more of the evolutionary tree will prove to be susceptible to specific strains of *B.t.*? The year of the publication or patent filing reporting pesticidal activity is shown in brackets. (Adapted from Reference 17.)



Diversity of *B.t.* Toxins

Regardless of the expression system used, its utility will depend heavily on the variety of *B.t.* toxin genes available. Consequently, there has been intense interest in recent years in accumulating, analyzing, and screening *B.t.* culture collections for novel pesticidal activities. Mycogen, Ecogen, Plant Genetic Systems (Ghent, Belgium), and the U.S. Department of Agriculture (Washington, DC) are among the organizations that now have large *B.t.* collections. For example, in our work with partners worldwide, Mycogen has accumulated a collection of more than 3000 *B.t.* strains derived from environmental samples from nearly 50 countries. As a result of this work, scientists now have a much better idea of how many *B.t.* genes there are, and how broad their utility is. Mycogen's collection, for example, is very diverse, as judged by (1) the range of toxin crystal morphologies; (2) toxin profiles assessed by SDS-PAGE; and (3) the broad range of subspecies serotypes (flagellar antigen subtypes) found (all known serotypes were found[6] and several new ones were discovered).

New *B.t.* Activities

Most *B.t.* strains characterized to date show activity against some species of the insect order Lepidoptera (caterpillars), Diptera (mosquitoes and blackflies), or Coleoptera (beetles), while many other crystal-producing strains have shown no

Why is a Raven like a Writing Desk?

Question: When are biopesticides like a video player?

Answer: When you think of the *B.t.* toxin gene as a universal cassette. Until relatively recently, *B.t.* was thought to be active only against a limited range of insect pests, notably caterpillars. That meant that *B.t.* pesticides were useful only for protecting a limited number of crops. Their markets were limited, just as the market for videoplayers would be if there were only a few esoteric films available on cassette.

B.t. may not be family entertainment yet, but its appeal is certainly broadening. Investigators are uncovering a broad range of strains active against flies, worms, mites and nematodes, among others. Clearly, this potentially opens up new markets. Furthermore, it provides greater variety in existing markets, which may help prevent the emergence of resistant pest populations.

Through molecular genetics (and the payment of an appropriate license fee to the patent owner, of course), the *B.t.* gene cassettes are available in formats to suit a variety of recombinant replay systems: *B.t.* itself, other bacteria (both dead and alive), and plants. Regardless of which format succeeds in which markets, and there will undoubtedly be intense competition, the increasing availability of new *B.t.* toxin genes seems to point strongly to an expanding total market for biological pest control.

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demonstrable toxicity to any other orders.

We have discovered a number of unusual biological activities. The first, in 1985, was the characterization of anti-Coleopteran activity from *B.t. san diego* subspecies *morrisoni*[7]. This strain, with a wafer-shaped crystal inclusion (see cover), was toxic to the Colorado Potato Beetle and resulted in Mycogen's first product, M-One (now largely superseded by M-Trak, its *Ps. fluorescens*-encapsulated counterpart). This was followed, a few years later, by the discovery of *B.t.* strains with activity against plant- and animal-parasitic nematodes[8], the first demonstration that some *B.t.* strains can kill noninsect pests.

The process has continued (Figure 1). Among the insects, we found activities against several Dipteran pests, including leafmining flies (Agromyzidae) and adult houseflies (*Musca domestica*). There are now also toxins active against "recalcitrant" Lepidoptera, such as beet armyworm (*Spodoptera exigua*). Novel strains and toxins were characterized by activity against commercially important beetle larvae (Coleoptera), including corn rootworm and soil grubs.

The discovery of strains specifically toxic to protozoan pathogens, animal-parasitic liver flukes (Trematoda), or mites (Acari) has broadened the potential *B.t.* product spectrum even further. With activities against unique targets, these novel strains retain their very high biological specificity: nontarget organisms remain unaffected. We now believe that it may be possible to find *B.t.*

Explaining *B.t.* diversity

Sporulating *B.t.* cells take on a huge metabolic burden while they accumulate protoxin: the rate of protoxin synthesis is about 33 to 45% of the overall rate of total protein synthesis[12]. Thus the ability to produce toxins must confer an important selective advantage. As for the pests, their survival will depend on developing resistance to the *B.t.* strains they encounter. One explanation of the enormous diversity of *B.t.* strains in nature might be that the bacterial genes that encode toxin proteins and the toxin-target genes in the host may evolve in tandem—coevolution[14]. As an insect becomes resistant to one strain of *B.t.*, a selection pressure for a different microbial strain may be created. That, in turn, may create pressure for a new insect variant.

Natural populations of *B.t.* are also diverse because the vast majority of toxin genes are on self-transmissable plasmids that allow their transfer between related cells to create novel combinations of toxins.

Classifying *B.t.* toxins

A large number of distinct *B.t* toxin genes have now been cloned and sequenced since the first one in 1985[13]. In 1989, Höfte and Whiteley[9] classified 42 *B.t.* crystal protein genes into 14 distinct genes, grouped into 4 major classes based on amino-acid sequence and host range. The classes were CryI (Lepidoptera-specific), CryII (Lepidoptera- and Diptera-specific), CryIII (Coleoptera-specific) and CryIV (Diptera-specific). Many more *B.t.* toxin genes have since been sequenced and analyzed. Following the analysis of the toxin domains of 29 distinct *B.t.* crystal proteins (Figure 2), we have added two new major classes to the nematode-active toxins[16], CryV and CryVI, to the Höfte and Whiteley classification. Several novel genes have also been added within the previously defined classes.

In some cases, there is apparently a good correlation between the structural similarity of toxin classes based purely on amino-acid sequence and on their corresponding activities. For example, the Lepidopteran-active CryI proteins appear to be closely related; all (with the notable exceptions of CryIB and CryIG) probably have a common ancestor. Excluding these two toxins, CryI proteins have greater than 62% sequence similarity to each other. Similarly, the Coleopteran-active CryIII proteins and CryIB have a common ancestral root of greater than 53% sequence similarity.

However, with the CryIV and the nematode-active proteins, the primary structure-function correlation becomes much more tenuous. For example, while CryIVA, B, and C have greater than 44% sequence similarity, CryIVD is more closely related to the CryII proteins. The nematode-active toxins fall into two completely unrelated groups, CryV and CryVI. While the CryV genes are related to other *B.t.* toxins in the "main tree," CryVI toxins appear to have a completely different root. Future analyses of the secondary and tertiary structures of toxin proteins may throw new light on their interrelatedness.

strains specific for virtually any pest target, from single-cell eukaryotes to the most advanced arthropod (see Figure 1) as long as: (1) the screened *B.t.* collection is sufficiently diverse; (2) the target organism can ingest (and, possibly, process) the toxins; (3) material is presented optimally; and (4) bioassays are developed.

Product Implications

How might the large, diverse, and growing collection of cloned and sequenced *B.t.* toxin genes be used in formulating new products? One possibility comes to mind from looking at the toxins present in existing nonrecombinant *B.t.*-based products.

Naturally occurring *B.t.* usually contain a number of plasmids encoding different toxins. What is interesting, however, is that commercial *B.t.* preparations contain toxins from widely divergent branches of the evolutionary tree. For example, Abbott Laboratories' (North Chicago, IL) Dipel for caterpillar control, which is derived from the *B.t. kurstaki* HD-1 strain, contains four toxin subtypes—CryIA(a), CryIA(b), CryIA(c), and CryIIA. Another Abbott product, Vectobac for mosquito control, contains CryIVA, CryIVB, CryIVD, and the 27-kDa CytA protein. CytA is a cytolytic protein, unrelated to any of the Cry proteins. This diversity in approved products encompasses a large proportion of the novel *B.t.* toxins classified in Figure 2.

Two aspects of this state of affairs are encouraging for manufacturers. The first is that first-generation, single-toxin, recombinant *B.t.* products will probably be followed by second-generation products containing multiple or distantly related toxins. With a greater diversity of toxins to choose from, second-generation products may prove both more effective and even less likely than current products to engender resistance in target populations. Second, the approval by regulatory bodies such as the EPA of existing commercial products containing a broad range of different *B.t.* toxins encourages the view that a wide range of *B.t.* toxins are considered safe and effective and that prod-

Plant or bacterial *B.t.*?

Recombinant microbial toxin delivery systems and transgenic plants probably have complementary roles to play in plant protection. On the one hand, the reengineering of the toxin gene and its genetic regulatory elements to permit sufficient expression in the plant host may limit the range of toxin genes that can be usefully expressed in plants. The same considerations may limit the use of multiple *B.t.* proteins in plants. Furthermore, the plant system has more inertia in that growers may find themselves "locked in" to a particular toxin gene product while microbial systems containing a new toxin or a complex range of toxins can be developed more quickly after new genes are discovered or created (plant products must be scaled-up over several growing seasons).

On the other hand, at the most practical level, the periodic application of microbial products to crops (perhaps more than once per growing season) represents an additional task for the farmer. And the initial microbial biotoxin level (of killed preparations, at least) will have to be higher to compensate for biodegrative and climatic losses between applications.

It is quite possible, therefore, that in some cases microbial insecticides may be the prevalent modality. In other circumstances, transgenic plants may confer economic advantages. In yet other situations, recombinant microbial and plant products will be used in tandem, the plants providing a background level of the more established *B.t.* toxins while the bacterial preparations are used to introduce variety and newer toxins.

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Table 1. Companies involved in *Bacillus thuringiensis* research, development, and production

Company	Focus	Commercial status of B.t.	No. of U.S. Patents issued (1-1-88 to 12-31-91)
Abbott	Nonengineered *B.t.*	Products	0
Agracetus/W.R. Grace	*B.t.* in plants	Laboratory	0
Agricultural Genetics	Nonengineered *B.t.*	Laboratory	1
Agrigenetics	*B.t.* in plants; relationship with Mycogen	Small-scale field trials	0
Boehringer-Mannheim	Licensor of *B.t.* technology	Products	4
Ciba-Geigy	Nonengineered *B.t.*, transconjugants, *B.t.* in plants	Products	0
Crop Genetics Int'l	Endophytic organisms expressing *B.t.* toxin	Small-scale field trials	0
DuPont	Agreement with Novo/Entotech on products	Products	1
Ecogen	Nonengineered *B.t.*, Transconjugants	Products	2
ICI	Nonengineered *B.t.*	Products	1
Kubota	Relationship with Mycogen in Asia	Small-scale field trials	0
Mitsubishi/Plantech	*B.t.* in plants	Laboratory	0
Monsanto	*B.t.* in plants	Small-scale field trials	0
Mycogen	Genetically engineered (dead) cells: CellCap	Products	22
Novo-Entotech	Nonengineered *B.t.*, classical mutagenesis	Large-scale field trials	1
Plant Genetic Systems	*B.t.* in plants	Small-scale field trials	0
Sandoz	Nonengineered and recombinant *B.t.*	Small-scale field trials	1
Shell	Relationship with Mycogen for CellCap products	Small-scale field trials	0

ucts containing multiple toxins are often desirable.

Future prospects

How will the diversity of *B.t.* toxins impact agriculture and society? One obvious benefit will be the addition of recombinant bacterial and plant products to the collection of "softer" environment-friendly tools in the armamentarium of integrated pest management[8]. The availability of large numbers of diverse *B.t.* toxins may enable better management of pest resistance, broaden the host range of biopesticides, increase their persistence in the field, and extend toxin delivery options. Pest control options outside agriculture (domestic, animal health, human health, etc.) will also be extended.

The family of *B.t.* toxins, which may include thousands of members, also represents a good system for exploring protein structure-function relationships in functionally related molecules. Numerous *B.t.* genes have been sequenced, novel activities found, and the three-dimensional structure of one *B.t.* toxin has been solved at high resolution (2.5) by X-ray crystallography[10]. Several laboratories are using this structure to model other molecules. On the one hand, this could lead to a deeper understanding of toxin receptors, the mode of toxin action, and the mechanisms of resistance in pests[11]. On the other, it could, by the end of the decade, lead to the development of chimeric, second-generation toxin molecules[12] and to advanced screening methods for discovering new toxins.

Of fifty-three U.S. patents on *Bacillus thuringiensis* granted in the last 21 years, 39 have been issued in the last 4 (Table 1). Two genetically engineered *B.t.* products have now been registered with the EPA and a third has been given an experimental-use permit. Thus, it is clear that rapid progress is being made in the basic science, invention, application, and commercialization of agricultural *B.t.*-based products.

Acknowledgments

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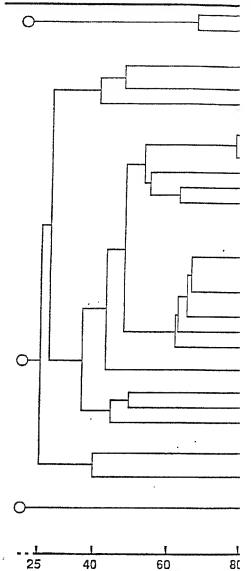
Fig. 2. Possible evolutionary relationships betwee
algorithm was used to compare amino-acid sequence



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Transgenic plants: an emerging approach to pest control.

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Insect pests are a major cause of damage to the world's commercially important agricultural crops. Current strategies aimed at reducing crop losses rely primarily on chemical pesticides. Alternatively transgenic crops with intrinsic pest resistance offer a promising alternative and continue to be developed. The first generation of insect-resistant transgenic plants are based on insecticidal proteins from Bacillus thuringiensis (Bt). A second generation of insect-resistant plants under development include both Bt and non-Bt proteins with novel modes of action and different spectra of activity against insect pests.

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